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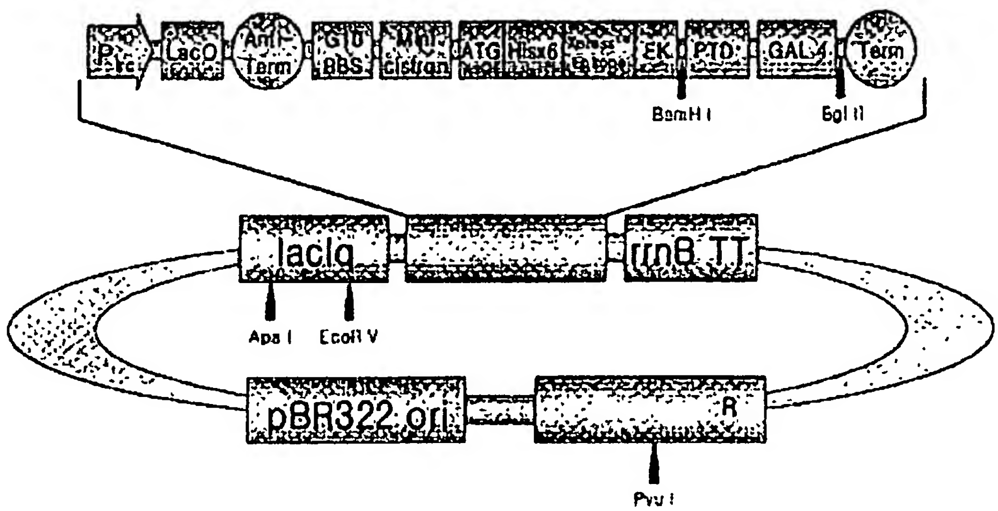
(54) Title: DNA/RNA TRANSDUCTION TECHNOLOGY AND ITS CLINICAL AND BASIC APPLICATIONS



PTD may be a sequence encoding a) Sim-2, b) Mph-1, c) Tat or d) R7.

(57) **Abstract:** This invention relates to a method for *in vivo* and *in vitro* transferring efficiently DNA/RNA coding materials regulating bio-function to cytoplasm or nucleus in eukaryotic or prokaryotic cells using PTD (Protein Transduction Domain) and DNA/RNA binding factor. Particularly, this invention provides a method for *in vivo* transferring the materials to cells through various routes comprising intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal, inhalation. Accordingly, the method of this invention can be used for technology to transfer DNA/RNA to various cell types and express them in the cells transiently or permanently in medicinal applications such as DNA/RNA vaccine and gene therapy, as well as basic applications.

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DNA/RNA TRANSDUCTION TECHNOLOGY AND ITS CLINICAL AND BASIC APPLICATIONS

TECHNICAL FIELD

This invention relates to a method for delivering DNA/RNA encoding a biological regulatory molecule, such as a biological regulator, *in vivo* or *in vitro* into eukaryotic or prokaryotic cytoplasm or nucleus, using PTD (Protein Transduction Domain) and DNA/RNA binding factor.

BACKGROUND ART

This invention relates in general to a system for effective transduction of biological regulatory molecules *in vivo* or *in vitro* into eukaryotic or prokaryotic cytoplasm or nucleus.

Typically, a living cell is known as being impermeable to macromolecules, for example proteins or nucleic acids. It is known as a crucial limiting factor for employing macromolecules in treatment, prevention and diagnosis of disease that only a small-size substance can pass through the plasmic membrane of a living cell in low rate while macromolecules, such as protein and nucleic acids etc, cannot permeate the cell membrane. For the treatment, prevention and/or diagnosis of disease, the biological regulatory molecules of interest should be delivered with their effective amount into the target cells. Until now, various methods for transferring biological regulatory molecules into target

cells, for example, by applying the molecules on the exterior or surface of the cells have been reported. Conventional means to deliver *in vitro* macromolecules into cells are as follows: electroporation, membrane fusion using liposomes, high-concentration projection using particular projectors coated with DNA, cultivation using calcium-phosphorous-DNA precipitate, DEAE-dextran transfection, infection of modified viral nucleic acid, direct injection to a single cell. However, these methods can deliver macromolecules to only a portion of the target cells, and can cause side effects to many other cells. Also, there are other methods to introduce *in vivo* macromolecules into cells: for example, scrape loading, calcium-phosphate precipitation, method using liposomes. However, a controversial matter is that the usage of these methods has *in vivo* limitations.

Therefore, a general and more efficient way to deliver *in vivo* and *in vitro* biologically active macromolecules into a cell without damaging it was required [L.A. Sternson, Ann. N.Y. Acad. Sci., 57, 19-21 (1987)]. For this purpose, chemical addition of lipid peptide [P. Hoffmann et al. Immunobiol., 177, 158-170(1988)] and a method using basic polymers such as polylysine or polyarginine [W-C. Chen et al., Proc. Natl. Acad. Sci., USA, 75, 1872-1876(1978)] were introduced, but these have not been clearly verified yet. Further, although folic acid [C.P. Leamon and Low, Proc. Natl. Acd. Sci., USA, 88, 5572-5576(1991)] was reported that it can move into a cell as folic acid-salt complex, it has not been verified yet whether it can be delivered even into the cytoplasm. Also, pseudomonas exotoxin was known as a kind of transporter [T. I. Prior et al., Cell, 64, 1017-1023(1991)]. Nevertheless, the effects resulted from the delivery of biologically active substances using the methods into the target cells are still unclear. In this regard, a novel method to deliver biologically active substances into target cells more safely and effectively is required continually.

As a result of study for the requirement, PTD (Protein Transduction Domain) was designed. Among various PTDs, the transcription factor, Tat, of Human Immunodeficiency Virus-1, HIV-1 has been studied well. This protein can pass through the cell membrane more effectively when it is organized by part of the amino acids distributed through 47 to 57 (YGRKKRRQRRR), where positively charged amino acids are distributed, than when it is in a complete form consisting of 87 amino acids [Fawell S. et al. Proc. Natl. Acad. Sci. USA 91, 664-668(1994)]. Like this, amino acids 267 to 300 of VP22 protein of Herpes Simplex Virus type 1 [Elliott G. et al. Cell, 88,223-233(1997)], amino acids 84 to 92 of UL-56 protein of HSV-2 (GeneBank code: D1047[gi:221784]), and amino acids 339 to 355 of ANTP (Antennapedia) protein of Drosophila [Schwarze S.R. et al. Trends Pharmacol Sci. 21, 45-48(2000)] are examples of other PTDs. Further, artificial peptides comprising positively charged amino acids also showed effects [Laus R. et al. Nature Biotechnol. 18, 1269-1272(2000)].

Thus, we, inventors, completed this invention by using fusion protein structured by fusing DNA/RNA binding factor or the DNA/RNA binding domain to PTD, and thereby significantly improving the delivery efficiency of a biological regulatory molecule of interest, such as DNA/RNA, into target cells. According to this invention, a biological regulatory molecule of interest can be introduced into target cells, tissues and organs, specifically or by the induction of specific stimulus.

DISCLOSURE OF INVENTION

The purpose of this invention is to provide a method for expressing a protein. For this purpose, a fusion protein is provided by combining DNA/RNA encoding

biological regulatory protein containing selectable DNA Binding Sequence, DBS; one or more homologous or heterologous binding protein, which comprises DNA/RNA binding factor or a part thereof (DNA Binding Domain, DBD) that can bind selectively to the DBS; and PTD at room temperature. Then, the DNA/RNA encoding regulatory protein is transferred *ex vivo* into strains or *in vivo* into each target organs through routes, such as intramuscular, intraperitonea, intravein, oral, nasal, subcutaneous, intradermal, mucosal and inhalation. In particular, when DNA/RNA encoding the biological regulatory protein comprises a promoter which regulates the expression of the DNA/RNA at a specific organ, tissue or cell, the biological regulatory protein can be expressed at a specific target site. Another purpose of this invention is to transduce one or more biological regulatory molecules selected from the group consisting of protein, DNA/RNA, fat, carbohydrate and chemical compound *in vitro* or *in vivo* into eukaryotic or prokaryotic cytoplasm or nucleus.

Furthermore, the purpose of this invention is to provide a novel method for gene therapy and DNA/RNA vaccine using the method of the present invention, and a method of transducing the interest DNA/RNA fragments into various kinds of prokaryotic and eukaryotic cells in order to perrmanently or transiently expressing the proteins with the interest DNA/RNA fragments.

BRIEF DESCRIPTIONS OF DRAWINGS

Fig. 1a to Fig. 1c show structures of recombinant expression vectors of this invention.

Fig. 2a and Fig. 2b are photographs of agarose gels after electrophoresis of the expression vectors of Fig. 1 digested with restriction enzymes.

Fig. 3 is a result of coomassie blue staining of the purified fusion protein expressed from the expression vectors.

Fig. 4 indicates the detection of CD8-z and Lck protein delivered into Jurkat T cells by Sim2-Gal4, Mph1-Gal4 and R7-Gal4 through western blot analysis using mAb of CD8 and Lck.

Fig. 5 indicates the detection of CD8-z and Lck protein delivered into Hela cells by Sim2-Gal4, Mph1-Gal4 and R7-Gal4 through western blot analysis using mAb of CD8 and Lck.

Fig. 6a to Fig. 6d indicate the detection of CD8-z and Lck expressed in the heart (Fig. 6a), liver (Fig. 6b), kidney (Fig. 6c) and spleen (Fig. 6d) of mouse using their mAb, after injecting pCD8-z-GBS and pLck-GBS with Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 through I.P.

Fig. 7a to Fig. 7c indicate specific expressions of each protein after injecting pL-CD8-z-GBS and pL-Lck-GBS with Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 through I.P.

Fig. 8a to Fig. 8c indicate specific expressions of each protein after injecting pL-CD8-z-GBS and pL-Lck-GBS with Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 through I.P.

In order to accomplish the above mentioned purposes, this invention provides a protein tranducing recombinant expression vector, comprising a fusion protein of PTD (Protein Transduction Domain) with one or more homologous or heterologous binding proteins having DNA/RNA Binding Domain (DBD) or DNA/RNA binding factor that is able to combine with specific DNA/RNA binding sequences, DNA encoding the binding proteins, DNA encoding the PTD, wherein the DNAs are operatively linked to an

expression regulatory sequence in the vector.

Moreover, the invention provides a recombinant expression vector, comprising a DNA/RNA encoding a biological regulatory protein containing DNA/RNA binding sequence at 3' or 5', which binds specifically to the DNA/RNA binding factor or the DNA/RNA binding domain, wherein the DNA/RNA is operatively linked to a promoter, as an expression regulatory sequence, that is cell, tissue or organ selective.

In addition, this invention provides a DNA structure comprising one or more biological regulatory molecules selected from the group consisting of protein, DNA/RNA, fats, carbohydrate and chemical compound, wherein the DNA structure is combined with DNA/RNA binding sequence that specifically binds to DNA/RNA binding factor or DNA/RNA binding domain by chemical or physical non-covalent or covalent bond.

Further, the invention provides a binding complex for delivering a biological regulatorory molecule of interest into cytoplasm or nucleus, comprising a fusion protein of PTD with one or more homologous or heterologous binding proteins that have DNA/RNA binding factor or DNA/RNA binding domain; and one or more biological regulatory molecules selected from the group consisting of protein, DNA/RNA, fats, carbohydrate and chemical compound are combined, wherein the biological regulatory molecule and the fusion protein are combined by chemical or physical non-covalent or covalent bond.

Moreover, this invention provides a binding complex to deliver DNA into the cytoplasm or the nucleus, comprising a fusion protein of PTD with one or more homologous or heterologous binding proteins containing DNA/RNA binding factors or DNA/RNA binding domain; and comprising a recombinant expression vector containing DNA/RNA binding sequence that specifically binds to the DNA/RNA binding factor or DNA/RNA binding domain, and DNA encoding a biological regulatory molecule, wherein

the DNA is operatively linked to an expression regulatory sequence in the vector.

In addition, using the fusion protein of PTD with the protein having DNA/RNA binding factor or DNA/RNA binding domain (DBD), this invention provides a method for expressing DNA/RNA encoding biological regulatory protein containing DBS, which is capable of selectively combining the binding factor or DBD, by delivering it into prokaryotic or eukaryotic cytoplasm or nucleus after contacting it with prokaryotic or eukaryotic cells through various routes including intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhalation *ex vivo* or *in vivo*.

Further, this invention provides a method for delivering a biological regulatory molecule to eukaryotic or prokaryotic cytoplasm or nucleus, comprising i) preparing a transducing recombinant expression vector which comprises DNA encoding PTD, and DNA encoding one or more homologous or heterologous binding proteins that contain DNA/RNA binding factor or DNA/RNA binding domain, wherein the DNA is operatively linked to an expression regulatory sequence in the vector; ii) obtaining a fusion protein by expressing the recombinant expression vector of i) in a host cell; iii) obtaining a binding complex by combining the fusion protein of ii) and one or more biological regulatory molecules selected from the group consisting of protein, DNA/RNA, fats, carbohydrate and chemical compound, through chemical or physical non-covalent or covalent bonds; and iv) mixed-culturing *ex vivo* the binding complex of iii) and cell culture or transferring *in vivo* the complex through routes including intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhalation.

This invention provides a method for delivering a biological regulatory molecule of interest to eukaryotic or prokaryotic cytoplasm or nucleus, comprising i) preparing a transducing recombinant expression vector which comprises DNA encoding PTD and

DNA encoding one or more homologous or heterologous binding proteins that contain DNA/RNA binding factor or DNA/RNA binding domain, wherein the DNAs are operatively linked to an expression regulatory sequence; ii) obtaining a fusion protein by expressing the recombinant expression vector of i) in a host cell; iii) preparing a recombinant expression vector which comprises DNA encoding a biological regulatory molecule, DNA/RNA binding sequence that binds specifically to the DNA/RNA binding factor or the DNA/RNA binding domain, wherein the DNA is operatively linked to an expression regulatory sequence; iv) obtaining a binding complex by combining the fusion protein obtained from ii) and the recombination expression vector of iii); and v) mixed-culturing *ex vivo* the binding complex of iv) and cell culture or transferring *in vivo* through routes including intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhalation.

By using the fusion protein of PTD and the binding protein containing DNA/RNA protein factor or DNA/RNA binding domain (DBD), the invention also provides a method of delivering a biological regulatory molecule, linked to DBS, which combines selectively with the above binding factor or DBD, by chemical or physical non-covalent or covalent bond, into prokaryotic or eukaryotic cytoplasm or nucleus by contacting it with prokaryotic or eukaryotic cells through various routes, such as intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhalation.

In this invention, "PTD" refers to a transportable peptide that delivers interest proteins, either directly linked by chemical or physical covalent or non-covalent bonds or indirectly linked using other linkers, into eukaryotic or prokaryotic cytoplasm or nucleus. PTD includes, but not limited to, Sim-2 [see, Chrast R. et al. Genome Res. 7, 615-624 (1997)], Mph1 [see, M.J Alkema et al.,Genes Dev. 11(2), 226-240(1997)], Tat [see,

Fawell S. et al. Proc. Natl. Acad. Sci. USA 91, 664-668(1994)], R7 (Cellgate, U.S.A.), SM5 (Dr. Quin, Vanderbilt University), VP22 [see, Elliott G. et al. Cell, 88,223-233(1997)], ANTP [see, Le Roux I. et al, Proc. Natl. Acad. Sci. USA 90, 9120-9124(1993)], Pep-1 and Pep-2 [see, May C. Morris et al, Nature Biotechnology, 19, 1173-1175(2001)].

"DNA/RNA binding factor" or "DNA/RNA binding domain (DBD)" refers to the whole protein or a part thereof which binds to specific DNA/RNA sequences. It includes for example transcriptional factor or viral protein.

"Binding protein" refers to a DNA/RNA binding factor or one or more homologous or heterologous the protein, which has DNA/RNA binding domain.

"Selective promoter" is a promoter which can express a gene encoding a protein in specific tissue, cell or organ - for example, T-cell-specific Lck, CD2 promoter and pancreas-specific insulin promoter. The promoter could be an inducible promoter.

The invention also provides a transducing recombinant expression vector which includes DNA encoding PTD and DNA encoding a protein having DBD.

The said transducing recombinant expression vector can be designed to comprise tag sequences which make it easy to purify the resulting fusion protein - for example, continuous histidine codon, hemaglutinine codon, Myc codon and maltose binding protein codon. Further, the vector include, but not limited to, cleavage site for removing unfavorable part from the fusion protein with restriction enzyme, such as enterokinase, factor X and thrombin, etc., expression controlling sequences and marker or reporter gene sequence for detecting the delivery.

As shown in the following examples, transducing recombinant expression vector of pPTD-GAL4, for example, pSim2-Gal4, pMph1-Gal4, pTat-Gal4 and pR7-Gal4, et.,

includes DNA encoding PTD, such as Sim-2, Mph-1, Tat and R7; six-His codon for the purification of protein expressed in a host cell; Asp-Asp-Asp-Asp-Lys sequence restricted specifically by enterokinase; and DNA encoding Gal4 DNA binding factor that binds to Gal4 binding sequence specifically.

The vector pPTD-Gal4 of this invention can be prepared by conventional PCR (polymerase chain reaction) simply using pTrcHisB (Invitrogen) as a template. Further, according to this invention, various kinds of the recombinant expression vector can be prepared by cutting out Gal4 gene (Invitrogen) from the vector using an appropriate restriction enzyme and replacing it with other DNAs encoding whole or a part of DNA binding factor which binds to specific DNA sequences. Gal4, as a DNA Binding Factor, is originally a transcription factor which is found in eukaryotes, prokaryotes and viruses. In one embodiment, Gal4 is employed for constructing fusion protein by combining it chemically or physically with a monoclonal antibody that specifically binds to a certain receptor and/or a ligand expressed on a specific cell, tissue or organ, in order to enhance the specific delivery. The substances to be fused with Gal4 comprise, but not limited to, protein fragments, fats, carbohydrates and their complexes. Gal4 fusion protein complex of this invention includes, but not limited to, DNA, RNA, carbohydrates, lipids or fats and chemical compounds linked to the transducing peptide chemically or physically.

In order to obtain a protein fused with transducing peptide, as a target protein, using the transducing recombinant expression vector, we transformed appropriate host cells, such as *E. coli,* with recombinant expression vector and obtained fusion protein expressed from those transformants, and then separated interest protein according to ordinary protocols for example poly Histidine and Ni^{2+}-NTA methods. The protein can be further purified, if necessary.

In addition, this invention provides a method for transducing a biological regulatory molecule, comprising i) obtaining binding complex by combining the biological regulatory molecule with transducing peptide or its derivatives, or with a fusion protein of transducing peptide and binding protein after activating them using binding inducer, and ii) delivering the interest biological regulatory molecule into cells by mixed-culturing the binding complexes with the cell culture. Furthermore, NLS (nuclear localization sequence) can additionally bind to PTD of fusion protein. The aforementioned binding inducers include reagents which link PTD (protein transducing domain) or fused protein of PTD and target protein, to the interest biological regulatory molecule (e.g. DNA, RNA, carbohydrates, fats, protein or chemicals) by physical or chemical means - for example, BMOE (Pierce Cat. No 22323) and DSP (Pierce Cat. No 22585), etc.

Moreover, when delivering a biological regulatory molecule, chemically or physically bound to a fusion protein of transducing peptide and binding protein, into a specific cell, tissue or organ cell, the binding protein can be mAb or its derivatives which bind specifically to receptors or ligands expressed in the target cell, tissue or organ. Meanwhile, the biological regulatory molecule can be a promoter and/or an enhancer that express in itself a gene in specific species, tissues, organs or cells.

Due to extremely small size, the transducing peptides in this invention are able to minimize the possible occurrence of biological interference with other biologically active substances. This invention will be described in more detail by the examples given below. However, it is intended that the examples are considered exemplary only and the scope of the invention is not limited.

EXAMPLES

Example 1: Preparation of Recombinant Expression Vector

Preparation of transducing recombinant expression vector for fusion protein of transducing peptide with binding protein having DBD (pSim2-Gal4, pMph1-Gal4, pTat-Gal4, pR7-Gal4, pCD8-z-GBS)

We, inventors, used Sim-2 gene (alanine at 558 ~ arginine at 566 from N terminus), Mph-1 gene (tyrosine at 858 ~ arginine at 868 from N terminus), Tat gene of HIV (tyrosine at 47 ~ arginine at 57 from N terminus), or base sequence encoding peptides consisting of 7 arginine amino acids as protein transducing peptides. We used Gal4 (Invitrogen) as binding proteins with DBD. In order to combine the above protein transducing peptides with base sequence encoding Gal4 to be bound to Gal4-binding sequence (GBS), SEQ ID NO: 1~4 corresponding to primers for Sim-2, Mph-1, Tat and 7 arginines, respectively, a primer of SEQ ID NO: 5 corresponding to 3' end of Gal4 to prepare the DNA structures and BamH I site for cloning were synthesized. And then, PCR was carried out with pfu turbo DNA polymerase (Stratagen) using the vector containing whole gene of the Gal4 protein (Clontech) as template.

In this example 1, SEQ ID NO: 1 is 5' primer for pSim2-gal4, SEQ ID NO: 2 is 5' primer for pMph1-Gal4, SEQ ID NO: 3 is 5' primer for pTat-Gal4, SEQ ID NO: 4 is 5' primer for pR7-Gal4, and SEQ ID NO: 5 is 3' primer for pSim2-Gal4, pMph1-Gal4, pR7-Gal4, and pTat-Gal4.

Preparation of the recombinant vector which contains DNA binding sequence (DBS) and encodes biological regulatory molecule (pLck-GBS, pINS-GBS, pL-CD8-z-GBS,

pL-LCK-GBS, pL-INS-GBS)

We prepared primers of SEQ ID NO: 6 and 7, wherein the sequences, expression vectors pCDNA-Lck or pCDNA-INS comprise a gene encoding Lck or insulin. Also, the sequences comprise Gal4 binding sequence (GBS) specifically binding to DNA binding sequence, Gal4, in restriction enzyme sites and Stu I at 5' and 3'. And then, we carried out PCR using pGAD as a template. The resulting reaction mixture from PCR was purified with the PCR purification kit (Qiagen) and digested with Bgl II and BamHI restriction enzymes for 48 hours. Then it was purified separately by 1% agarose gel elecrophoresis, and stained with ethidium bromide.

Also, each DNA of Lck-GBS, INS-GBS and CD8-z-GBS was separated from pLck-GBS, pINS-GBS and pCD8-z-GBS with restriction enzymes and cloned with the expression vector pLck-Luc which is selectively expressed in T cell. The recombinant expression vector prepared through cloning in the above method was named, respectively, pSim2-Gal4(a), pMph1-Gal4(b), pTat-Gal4(c), pR7-Gal4(d), pCD8-z-GBS(e), pLck-GBS(f), pINS-GBS(g), pL-CD8-z-GBS(h), pL-Lck-GBS(i), and pL-INS-GBS(j) and their structures are shown in Fig. 1a-1c.

SEQ ID NO: 6 is base sequence of 5' primer for preparation of Gal4 Binding Sequence (GBS), and SEQ ID NO: 7 is base sequence of 3' primer for preparation of Gal4 Binding Sequence.

Example 2: Preparation of *E. coli* Transformant and Expression and Purification of Fusion Protein

E. coli DH5 (ATCC No. 53863) was transformed with the expression vectors,

pSim2-Gal4(a), pMph1-Gal4(b), pTat-Gal4(c) and pR7-Gal4(d) prepared in Example 1 using heat shock transformation. Then, 2ml of the transformant was inoculated to 100ml of LB medium and pre-cultured with agitation at 37℃ for 12 hours. Next, after the resulting culture was inoculated to 1000ml of LB medium and cultured at 37℃ for 4 hours, the expression of lac operon was induced by adding 1mM of IPTG (Isopropyl-D-thiogalactopyranoside, GibcoBRL cat. # 15529-019). Subsequently, it was cultured for another 8 hours to induce the expression of fusion protein. The above culture was centrifuged at 6,000rpm at 4℃ for 20 minutes to remove the supernatant. The remaining pellets were dissolved in 10ml of buffer solution 1 (50mM NaH_2PO_4, 300mM NaCl, 10mM imidazole, pH 8.0) containing 1㎎/㎖of lysozyme (Sigma, cat.# L-7651) and placed on ice for 30 minutes. Then, the solution was treated with supersonic waves with the intensity of 300W for 10 seconds using a supersonic homogenizer (Heat System, Ultrasonic Processor XL), and then chilled for 10 seconds. This was repeated so that the total cumulated time for supersonic wave exposure was 3 minutes. The effluent was centrifuged at 12,000rpm at 4℃ for 20 minutes to remove the fragments of the debris and separate only the pure effluent. 2.5㎖of 50% Ni^{2+}-NTA agarose slurry (Qiagen, cat# 30230) was added to the effluent and mixed for 1hour at 200rpm at 4℃ to combine the fusion protein with Ni^{2+}-NTA agarose. This mixture was put through a 0.8 x 4 cm chromatography column (BioRad, cat. # 731-1550). The fusion protein was washed twice with 4ml of buffer solution 2 (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, pH 8.0) and then fractioned 4 times using 0.5㎖ of buffer solution 3 (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, pH 8.0). Fig. 3 shows the result of a coomassie blue staining after SDS-PAGE was carried out. In Fig. 3, lane 1 means the standard molecular weight of protein and Sim2-Gal4(a), Mph1-Gal4(b), Tat-Gal4(c) and R7-Gal4(d) are

showed, respectively.

Example 3: Delivery and Expression of the DNA into Jurkat T cell by Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 (*in vivo*)

After combining the fusion protein of Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 resulted from Example 2 with linear DNA structure pCD8-z-GBS, pLck-GBS and pINS-GBS at 37℃, 1ml of Jurkat cells (ATCC No. TIB-152) were added to 35mm Petri dish and reacted at 37℃ for 30 minutes. The reaction was terminated and collected cells, and the cells were reacted in 100ml of elution buffer solution [0.2% triton X-100, 150mM NaCl, 10mM Tris-HCl, 400 M EDTA, 1mM Na_3VO_4, 10mM NaF, 1mM PMSF, 10g aprotinin, 10g leupeptin] at 4℃ for 30 minutes and then centrifuged at 14,000rpm for 15 minutes to obtain the cell elution solution.

This cell elution solution was separated with SDS-PAGE gel, and the expressed protein was detected through Western Blot analysis using mAb (OKT8) for CD8, mAb for Lck, mAb for INS. The result is shown in Fig. 4 (the result of INS is not shown). In Fig. 4, the first lane represents the standard molecular weight of protein and delivery by Sim-2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

Example 4: Delivery and Expression of DNA into Hela cell by Sim2-Gal4, Mph1-Gal4, Tat-Gal4, or R7-Gal4 (*in vitro*)

As disclosed in Example 3, pCD8-z-GBS, pLck-GBS and pINS-GBS combined with Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 were delivered to the Hela cells. Then, CD8-z, Lck and insulin (INS) expressed in the cell were detected using Western Blot analysis. The results except for insulin are shown in Fig. 5. In Fig. 5, lane 1 means the standard molecular weight of protein, and delivery by Sim2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

Example 5: Delivery and Expression of DNA by Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 (*in vivo*)

Fusion proteins of Sim-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4 prepared in Example 4 and pCD8-z-GBS, pLck-GBS and pINS-GBS were fused to form binding protein complexes. 0.5mg/ml of each of the prepared complexes was injected by I.P. into C57B6 mouse. After 4 hours, several organs, heart, liver and spleen, are extracted. Western blot analysis methods were employed to detect CD8-z, Lck and insulin expressed on the surface due to this protein complex. The results except for insulin are shown in Fig. 6a to 6d.

i) Heart (Fig. 6a): The first lane is standard molecular weight of protein and delivery by Sim2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

ii) Liver (Fig. 6b): The first lane is standard molecular weight of protein and delivery by Sim2-Gal4: CD8-z(a), Lck(e); delivery by Mph1-Gal4: CD8-z(b), Lck(f);

delivery by Tat-Gal4: CD8-z(a), Lck(g); and delivery by R7-Gal4: CD8-z(d), Lck(h), are showed, respectively.

iii) Kidney (Fig. 6c): The first lane is standard molecular weight of protein and delivery by Sim2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

iv) Spleen (Fig. 6d): The first lane is standard molecular weight of protein and delivery Sim2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

Example 6: Cell-specific expression of target DNA *in vivo* by Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-Gal4

The plasmids prepared from Example 1, pL-CD8-z-GBS, pL-Lck-GBS and pL-INS-GBS, were linearized followed by being fused with Sim2-Gal4, Mph1-Gal4, Tat-Gal4 and R7-INS-Gal4 proteins, respectively, in order to obtain fusion protein complexes around 37℃. 0.5mg/ml of each of the prepared complexes was injected by I.P. into C57B6 mouse. 4 hours later, liver, T-cells and B-cells were extracted, and western blot analysis methods were used to detect CD8-z, Lck, and insulin expressed on the surface due to this protein complex. The results of these experiments except for insulin are shown in fig. 7a to 7c.

i) T cell (Fig. 7a): The first lane is standard molecular weight of protein and delivery by Sim2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f);

delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

ii) B cell (Fig. 7b): The first lane is standard molecular weight of protein and delivery by Sim2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

iii) Liver cell (Fig. 7c): The first lane is standard molecular weight of protein and delivery by Sim2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

0.5mg/ml of each of the obtained complexes of the fusion proteins, Sim2-Gal4, Mph-Gal4, Tat-Gal4 and R7-Gal4, and DNA structures, pL-CD8-z-GBS, pL-Lck-GBS and pL-INS-GBS was injected epithermally to C57B6 mouse. Six hours later, liver, T-cells and B-cells are extracted, and western blot analysis methods are used to detect expressions of CD8-z, Lck, and insulin with their mAbs. The results of these experiments are shown in fig. 8a to 8c.

i) T cell (Fig. 8a): The first lane is standard molecular weight of protein and delivery by Sim-2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

ii) B cell (Fig. 8b): The first lane is standard molecular weight of protein and delivery by Sim-2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

iii) Liver cell (Fig. 8c): The first lane is standard molecular weight of protein and delivery by Sim-2-Gal4: CD8-z (a), Lck (e); delivery by Mph1-Gal4: CD8-z (b), Lck (f); delivery by Tat-Gal4: CD8-z(c), Lck (g); and delivery by R7-Gal4: CD8-z (d), Lck (h) are showed, respectively.

INDUSTRIAL APPLICABILITIES

This invention relates to a technology that can deliver DNA effectively into cytoplasm or nucleus of eukaryotic or prokaryotic cell through various routes including intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal or inhalation, using DNA/RNA structure containing DNA/RNA binding factor which can be combined to PTD or specific DNA/RNA sequence, fusion protein which can be fused with binding protein that has DNA/RNA binding domain or DNA/RNA binding sequence which is specifically combined with biological regulator and DNA/RNA binding factor. This technology can be used to not only practical application for development of DNA/RNA vaccine and gene therapy, but also basic research that investigate function of protein which is expressed inside of cell continuously or temporarily by certain gene.

WHAT IS CLAIMED IS:

1. A binding complex for delivering DNA/RNA into cytoplasm or nucleus, comprising a fusion protein of PTD and one or more homologous or heterologous binding proteins having DNA/RNA binding factor or DNA/RNA binding domain; and a DNA/RNA binding sequence which is specifically bound to the DNA/RNA binding factor or the DNA/RNA binding domain, and DNA/RNA encoding biological regulatory molecule.

2. The binding complex according to claim 1, wherein NLS (Nuclear Localization Sequence) is additionally combined with the PTD of fusion protein.

3. The binding complex according to claim 1 or claim 2, wherein PTD is selected from the group consisting of Mph-1, Sim-2, Tat, R7, VP22, ANTP, MTS, Pep-1, and Pep-2.

4. The binding complex according to claim 1 or claim 2, wherein the biological regulatory molecule is a promoter or an enhancer that specifically expresses a gene in specific species, tissues, organs or cells.

5. The binding complex according to claim 4, wherein the promoter is an inducible promoter or an enhancer.

6. The binding complex according to claim 1 or claim 2, wherein the complex is delivered *in vivo* into cytoplasm or nucleus through routes including intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal and inhalation.

7. A method for delivering a biological regulatory molecule into eukaryotic or prokaryotic cytoplasm or nucleus, comprising:

i) preparing a transducing recombinant expression vector which comprises DNA encoding PTD and DNA encoding one or more homologous or

heterologous binding proteins that contain DNA/RNA binding factor or DNA/RNA binding domain, wherein the DNA is operatively linked to an expression regulatory sequence in the vector;

ii) obtaining a fusion protein by expressing the recombinant expression vector of i) in a host cell;

iii) obtaining a binding complex by combining the fusion protein of ii) and one or more biological regulatory molecules selected from the group consisting of protein, DNA/RNA, fats, carbohydrate and chemical compound, through chemical or physical non-covalent or covalent bonds; and

iv) mixed-culturing *ex vivo* the binding complex of iii) and cell culture or transferring *in vivo* the complex through routes including intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal and inhalation.

8. A method for delivering a biological regulatory molecule into eukaryotic or prokaryotic cytoplasm or nucleus, comprising:

i) preparing a transducing recombinant expression vector which comprises DNA encoding PTD and DNA encoding one or more homologous or heterologous binding proteins that contain DNA/RNA binding factor or DNA/RNA binding domain, wherein the DNAs are operatively linked to an expression regulatory sequence;

ii) obtaining a fusion protein by expressing the recombinant expression vector of i) in a host cell;

iii) preparing a recombinant expression vector which comprises DNA

encoding a biological regulatory molecule, DNA/RNA binding sequence that binds specifically to the DNA/RNA binding factor or the DNA/RNA binding domain, wherein the DNA is operatively linked to an expression regulatory sequence;

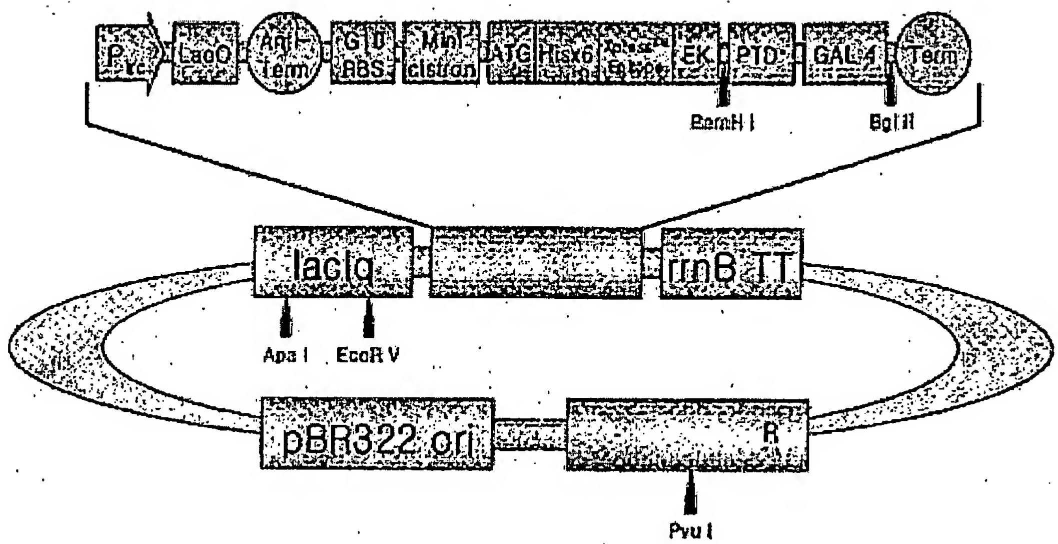
iv) obtaining a binding complex by combining the fusion protein obtained from ii) and the recombination expression vector of iii);

V) mixed-culturing *ex vivo* the binding complex of iv) and cell culture or transferring *in vivo* the complex through routes including intramuscular, intraperitoneal, intravein, oral, nasal, subcutaneous, intradermal, mucosal and inhalation.

9. The method according to claim 7 or claim 8, wherein ii) comprises further combining NLS (Nuclear Localization Sequence) with PTD of fusion protein.

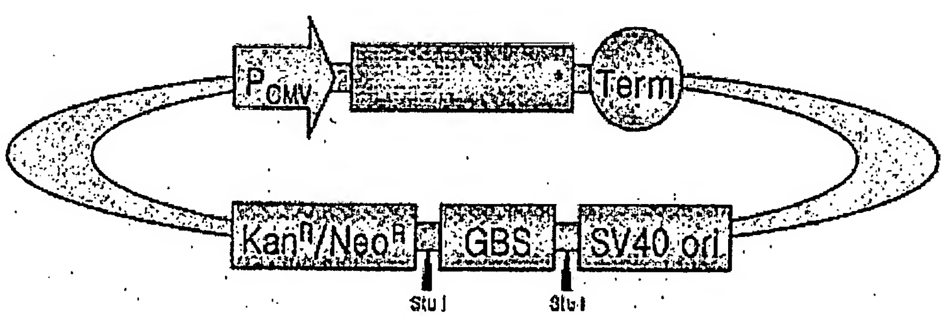
10. A protein transducing recombinant expression vector, comprising DNA encoding PTD and DNA encoding one or more homologous or heterologous binding proteins having DNA/RNA binding factor or DNA/RNA binding domain, wherein the DNA is operatively linked to an expression regulatory sequence in the vector.

Fig. 1a



PTD may be a sequence encoding a) Sim-2, b) Mph-1, c) Tat or d) R7.

Fig. 1b



Target gene consists of sequences encoding e) CD8-z, f) Lck.

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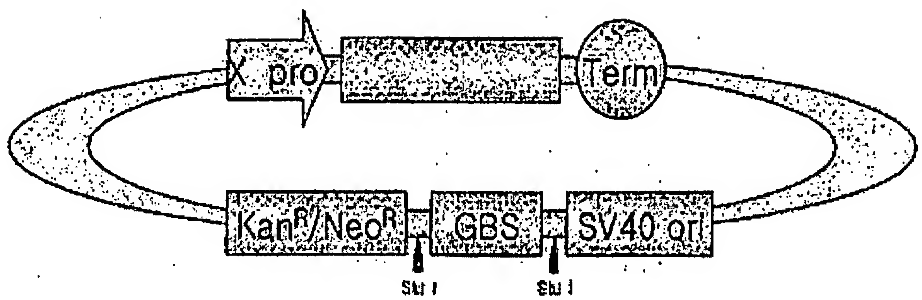
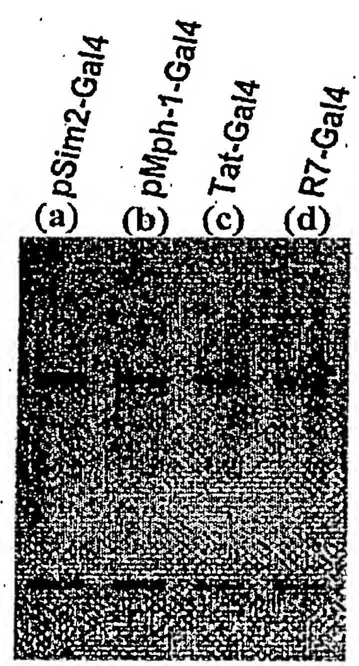
Fig. 1c



X pro indicates a human CD2 promoter which can regulate a T cell-specific expression, and Target gene consists of sequences encoding g) CD8-z, h) Lck.

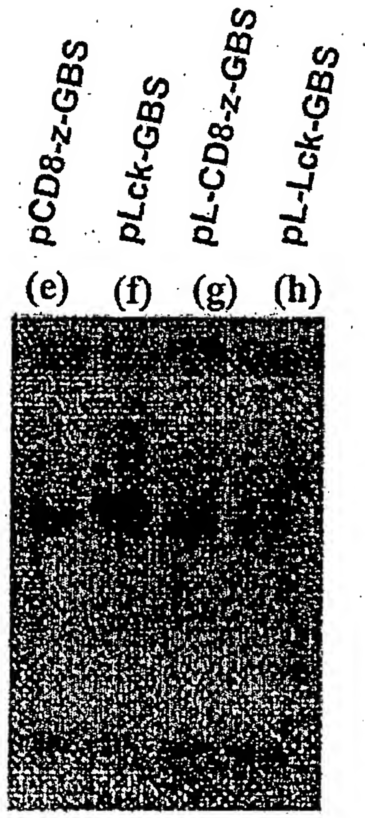
Fig. 2a



10/534433

3/9

Fig. 2b



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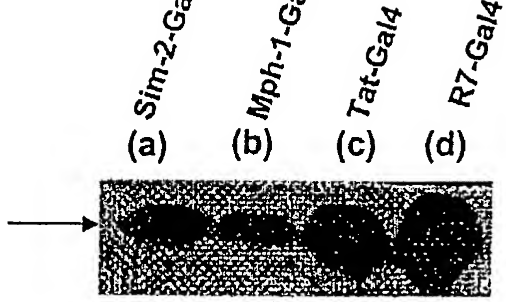

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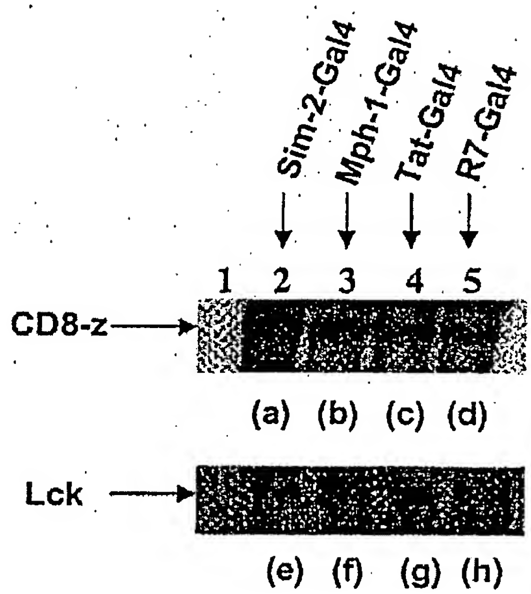

Sim-2-Gal4
Mph-1-Gal4
Tat-Gal4
R7-Gal4
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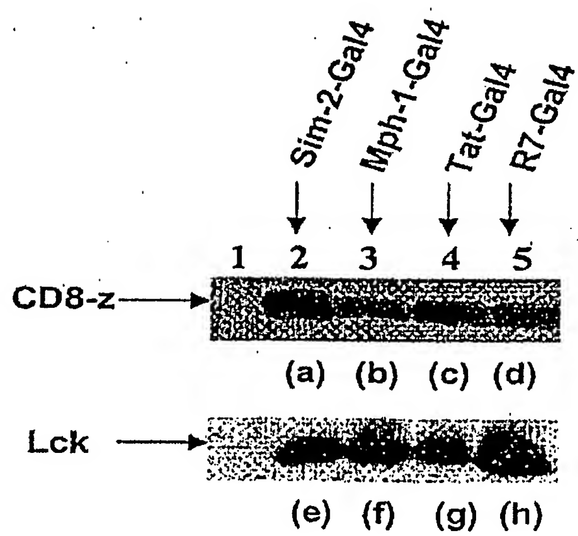
CD8-z
(a) (b) (c) (d)
Lck
(e) (f) (g) (h)

Fig. 5



Sim-2-Gal4
Mph-1-Gal4
Tat-Gal4
R7-Gal4
1 2 3 4 5
CD8-z
(a) (b) (c) (d)
Lck
(e) (f) (g) (h)

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Fig. 6a

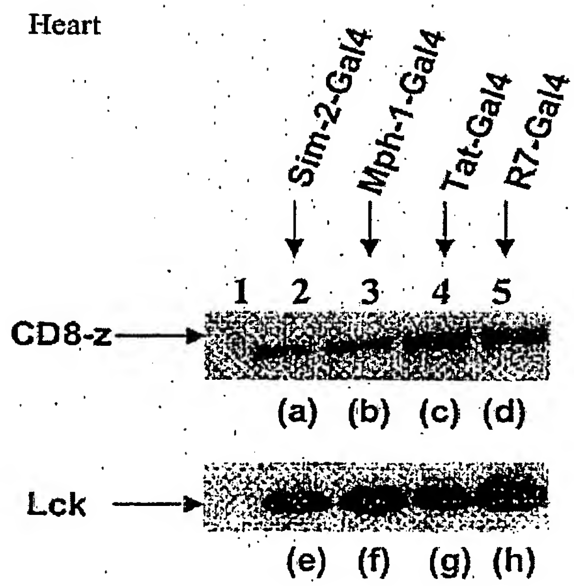

Fig. 6b

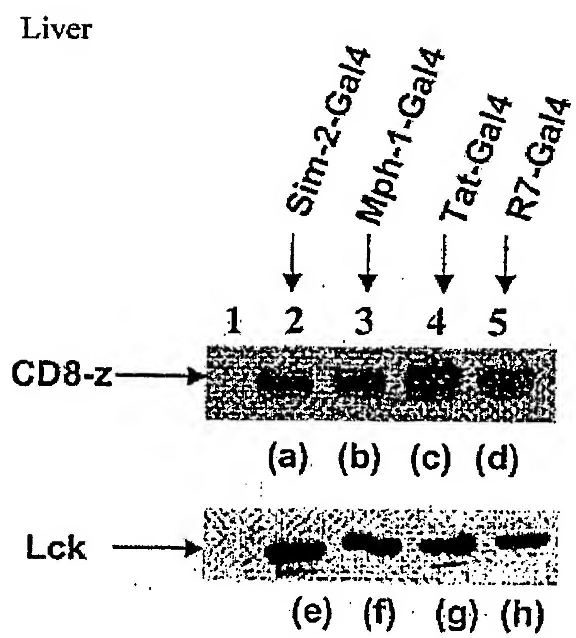

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Fig. 6c

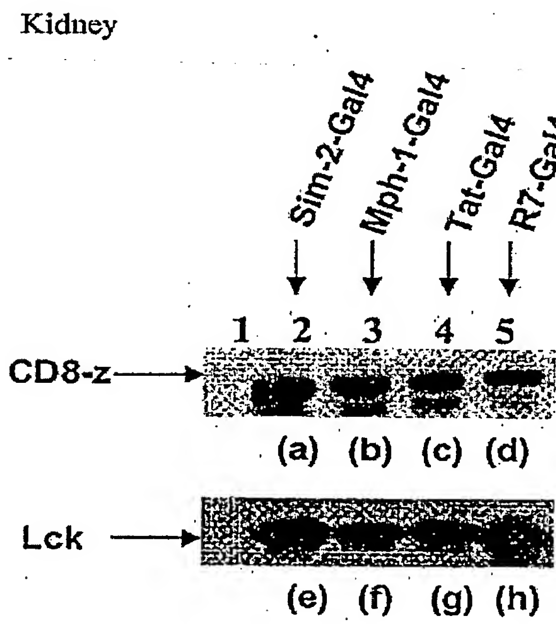


Fig. 6d

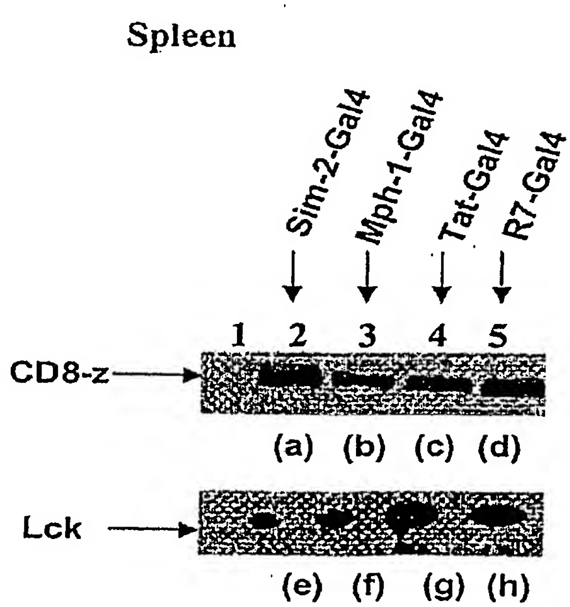
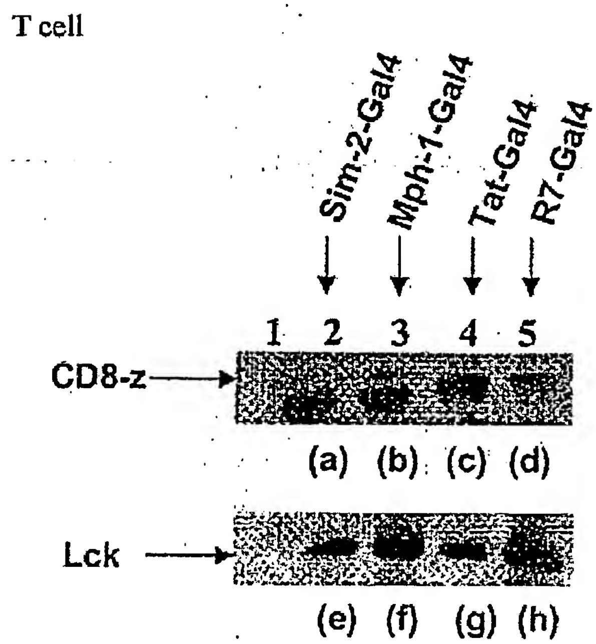


Fig. 7a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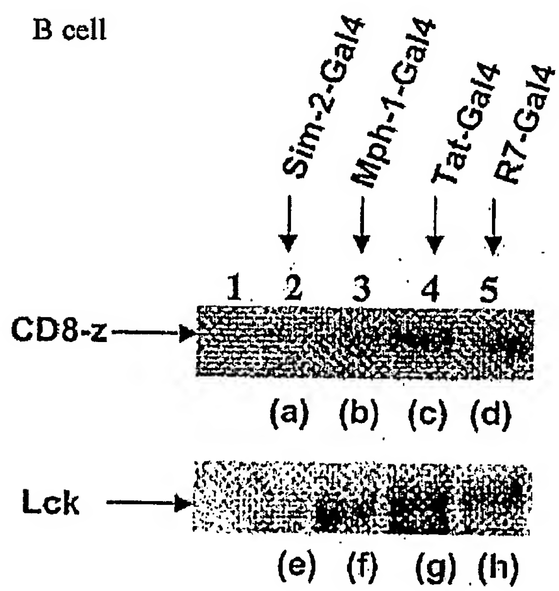
Mph-1-Gal4
Tat-Gal4
R7-Gal4
1 2 3 4 5
CD8-z
(a) (b) (c) (d)
Lck
(e) (f) (g) (h)

Fig. 7b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Mph-1-Gal4
Tat-Gal4
R7-Gal4
1 2 3 4 5
CD8-z
(a) (b) (c) (d)
Lck
(e) (f) (g) (h)

10/534433

Fig. 7c

Liver cell

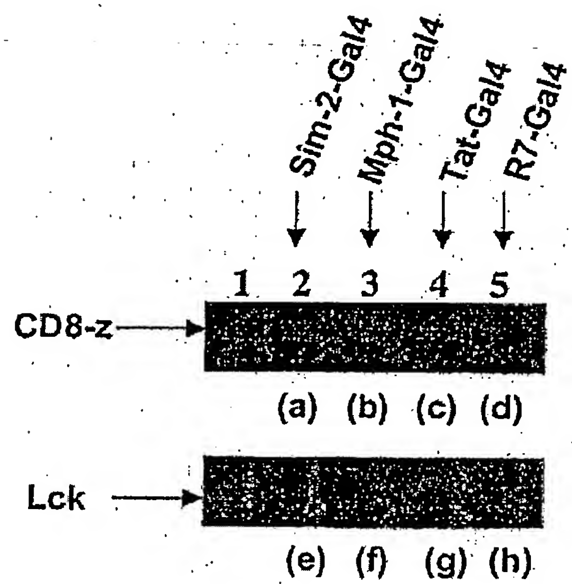
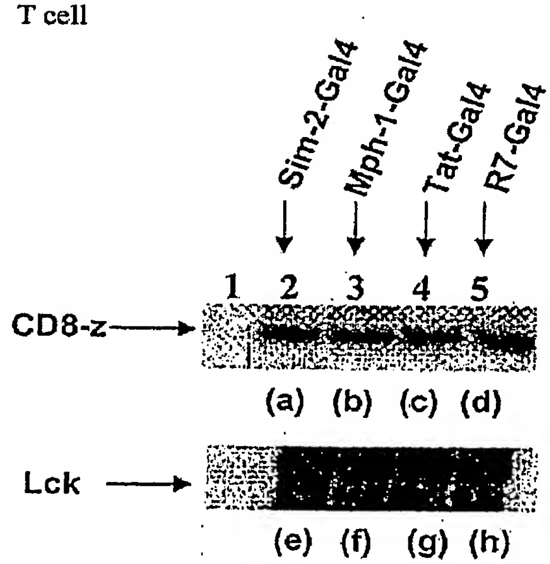


Fig. 8a

T cell



10/534433

Fig. 8b

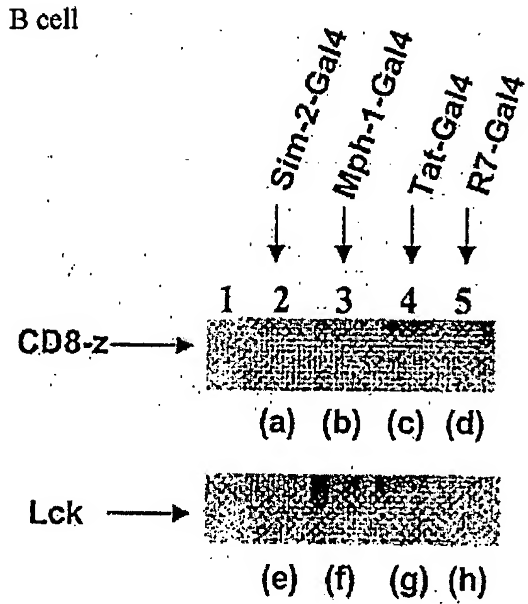
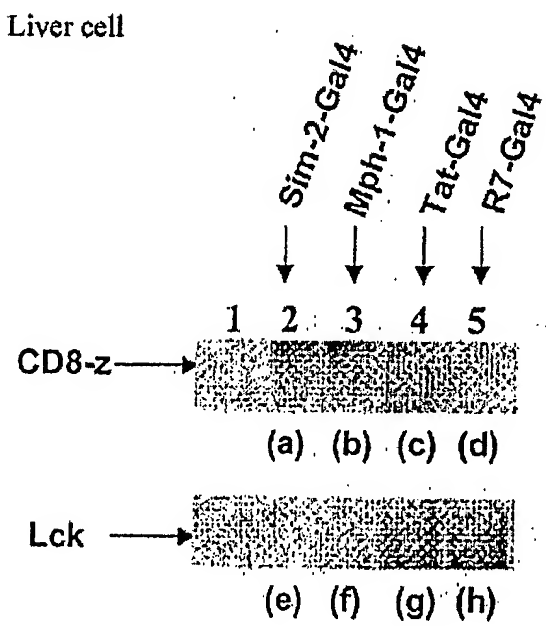


Fig. 8c



10/534433
JC14 Rec'd PCT/PTO 10 MAY 2005

SEQUENCE LISTING

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<120> DNA/RNA TRANSDUCTION TECHNOLOGY AND ITS CLINICAL AND BASIC APPLICATIONS

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2003/002425

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C07K 19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C07K 19/00, C07K 7/06, C12N 15/00, C12N 15/62, C12N 15/63

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

NCBI PubMed, Esp@cenet, CA "GAL4, Protein transduction domain, DNA binding domain, fusion protein, DNA delivery"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YE D. et al., 'Evaluation of strategies for the intracellular delivery of proteins', In: Pharm. Res., September 2002, Vol. 19(9), pp. 1302-1309. See the whole document	1-10
Y	CHAN C. K. et al., 'Mutual exclusivity of DNA binding and nuclear localization signal recognition by the yeast transcription factor GAL4: implications for nonviral DNA delivery', In: Gene Ther., 1998, Vol. 5(9), pp. 1204-1212. See the whole document	1-10
Y	Daviet L. et al., 'Identification of limiting steps for efficient trans-activation of HIV-1 promoter by Tat in Saccharomyces cerevisiae', In: J. Biol. Chem., 1998, Vol. 273(43), pp. 28219-28228. See the whole document	1-10
Y	WO 1999/010376 A1 (Washinton Uni.) 4 March 1999 See the claims	1-10
A	Eguchi A. et al., 'Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells', In: J. Biol. Chem., 2001, Vol. 276(28), pp. 26204-26210. See the whole document	1-10

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 FEBRUARY 2004 (20.02.2004)	20 FEBRUARY 2004 (20.02.2004)

Name and mailing address of the ISA/KR	Authorized officer
Korean Intellectual Property Office 920 Dunsan-dong, Seo-gu, Daejeon 302-701, Republic of Korea	CHO, YOUNG GYUN
Facsimile No. 82-42-472-7140	Telephone No. 82-42-481-8132

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2003/002425

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material
      - [X] a sequence listing
      - [ ] table(s) related to the sequence listing

   b. format of material
      - [X] in written format
      - [X] in computer readable form

   c. time of filing/furnishing
      - [X] contained in the international application as filed
      - [X] filed together with the international application in computer readable form
      - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR2003/002425

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 1999/010376 A1	04.03.1999	EP 1005486 A1 JP 2001513987 T	07.06.2000 11.09.2001